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EphA2 is a key effector of the MEK/ERK/RSK pathway regulating glioblastoma cell proliferation

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Abstract

EphA2, a member of the Eph receptor tyrosine kinases, is frequently overexpressed in a variety of malignancies, including glioblastoma, and its expression is correlated with poor prognosis. EphA2 acts as a tumor promoter through a ligand ephrin-independent mechanism, which requires phosphorylation of EphA2 on serine 897 (S897), leading to increased cell migration and invasion. In this study, we show that ligand-independent EphA2 signaling occurs downstream of the MEK/ERK/RSK pathway and mediates epidermal growth factor (EGF)-induced cell proliferation in glioblastoma cells. Suppression of EphA2 expression by long-term exposure to ligand ephrinA1 or EphA2-targeted shRNA inhibited EGF-induced cell proliferation. Stimulation of the cells with EGF induced EphA2 S897 phosphorylation, which was suppressed by MEK and RSK inhibitors, but not by phosphatidylinositol 3-kinase (PI3K) and Akt inhibitors. The RSK inhibitor or RSK2-targeted shRNA also suppressed EGF-induced cell proliferation. Furthermore, overexpression of wild-type EphA2 promoted cell proliferation without EGF stimulation, whereas overexpression of EphA2-S897A mutant suppressed EGF- or RSK2-induced proliferation. Taken together, these results suggest that EphA2 is a key downstream target of the MEK/ERK/RSK signaling

pathway in the regulation of glioblastoma cell proliferation.

Keywords: EphA2, RSK, EGF, cell proliferation, glioblastoma

1. Introduction

Glioblastoma is the most frequent malignant brain tumor, and is also one of the most lethal human cancers. Signaling from receptor tyrosine kinases is known to play key roles in regulating cell proliferation, survival, migration, and metabolism, and amplification and mutations of those kinases are major factors in development and progression of glioblastoma. Among them, epidermal growth factor (EGF) receptor is the most frequently mutated genes in glioblastoma [1, 2]. EGF receptor mutations and amplification alter the activities of downstream signaling pathways, including the Ras/Raf/MEK/ERK pathway and the PI3K/Akt signaling pathway, both of which contribute to the regulation of cell proliferation, survival, and motility [3].

The p90 ribosomal S6 kinase (RSK) family of serine/threonine protein kinases is directly activated by ERK1 and ERK2 downstream of tyrosine kinase receptors including EGF receptor. In human, four RSK isoforms (RSK1-4) have been identified, and their unique structural features are the presence of two distinct functional kinase domains. They share a high degree of sequence homology, especially in the two kinase domains [4-6]. RSKs are implicated in a variety of cellular functions, including cell proliferation and migration, through phosphorylation of

various cytosolic and nuclear targets. RSKs are also involved in the regulation of cancer cell proliferation and invasion, and emerge as potential therapeutic targets in various types of human cancer [5-7]. In glioblastoma, a marked increase of RSK2 expression in patient samples compared to that in normal brain tissues was observed [8], although its role in glioblastoma cells is not fully understood.

Eph receptors constitute the largest family of receptor tyrosine kinases. Their ligands, ephrins, are membrane-anchored proteins, and ephrin-Eph receptor interactions caused by cell-cell contact initiate tyrosine phosphorylation and signaling of Eph receptors via homotypic Eph-Eph interactions and regulate cell proliferation and migration during development and in tissue homeostasis [9-10]. Many studies have shown that dysregulation of ephrin/Eph receptor signaling contributes to cancer progression. Among them, EphA2 is frequently overexpressed in a variety of human cancers including glioblastoma [11-13]. EphA2 is highly expressed in specimens of glioblastoma but not in normal brain, and increased expression of EphA2 has been shown to correlate with poor survival of patients with glioblastoma [14-16]. Previous studies have reported that EphA2 mediates ligand ephrin-independent promotion of cell migration and invasion, whereas stimulation of EphA2 with ligand

ephrinA1 suppresses cell migration [17-21]. EphA2 has been also shown to contribute to the maintenance of stem-like tumor-propagating cells in glioblastoma [22, 23]. Phosphorylation of EphA2 on serine 897 (S897) by Akt plays a key role in EphA2 ligand-independent signaling. In contrast, ligand ephrinA1 stimulation induces dephosphorylation of EphA2 on S897 and suppresses EphA2 ligand-independent effects [17, 24]. However, a recent study reported that RSK phosphorylates S897 of EphA2 in various cancer cell types and promotes cell migration and invasion [25]. In the present study, we provide evidence that EGF induces phosphorylation of EphA2 on S897 through the MEK/ERK/RSK pathway and promotes glioblastoma cell proliferation. Our results suggest that EphA2 serves as a key RSK substrate for the regulation of glioblastoma cell proliferation and cancer progression.

2. Materials and Methods

2.1. Plasmids

Wild-type EphA2 (EphA2-WT) and S897A mutant (EphA2-SA) were subcloned into pcDNA3 vector (Life Technologies) as described previously [21, 24]. The YFP expression vector (pCAG) [26] was a generous gift from Drs. J. Miyazaki (Osaka University, Osaka, Japan) and T.

Saito (Chiba University, Chiba, Japan). Human RSK2 was obtained from U-251 cells and subcloned into pcDNA3 with a HA tag sequence at the N-terminus. The nucleotide sequence was confirmed after construction using the ABI Prism 310 Genetic Analyzer. We used a double promoter vector, encoding YFP and a short hairpin RNA (shRNA) for control luciferase (shControl) or EphA2 (shEphA2) to express YFP protein and the shRNA in the same cells, as described previously [27]. The shRNA for human RSK2 was designed to target 21 nucleotides of the human RSK2 transcript (5'-gggaggagatttgtttacacg-3' [25]) and expressed using pSilencer-hygro (Life Technologies).

2.2. Reagents and Antibodies

The pharmacological MEK inhibitors U0126 and PD98059, and phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 were purchased from Merck Millipore. Akt inhibitor MK-2206 was from ChemieTek, and RSK inhibitor BI-D1870 was from Santa Cruz Biotechnology. We used the following antibodies in this study: a rabbit polyclonal antibody against EphA2 (C-20) (Santa Cruz Biotechnology); a mouse monoclonal antibody against α-tubulin (Sigma); rabbit monoclonal antibodies against S897 phospho-EphA2 (D9A1) and T308 phospho-Akt (C31E5E), rabbit

polyclonal antibodies against Akt, ERK, and T202/Y204 phospho-ERK, a mouse monoclonal antibody against EphA2 (8B6) (Cell Signaling Technology); a mouse monoclonal antibody against HA (3F10) (Millipore); secondary antibodies conjugated to horseradish peroxidase (DAKO); Alexa Fluor 488-conjugated anti-GFP and Alexa Fluor 594-conjugated goat anti-mouse IgG (Thermo Fisher Scientific).

2.3. Cell culture and transfection

U-251 cell line was obtained from European Collection of Cell Cultures (ECACC). A172 cell line was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. U-251, A172, and HEK293T cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 4 mM glutamine, 100 units/ml of penicillin, and 0.1 mg/ml of streptomycin under humidified air containing 5% CO_2 at 37ºC. Cells were transfected with indicated expression vectors using polyethyleneimine MAX.

2.4. MTT assay

Cell proliferation was assessed using the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

U-251 cells were seeded onto 96-well plates (2500 cells/well) in serum-free medium for 15 h. Then cells were treated with 100 ng/ml recombinant human EGF (Sigma) together with 1 μg/ml control human Fc (Jackson ImmunoResearch Laboratories) or ephrinA1-Fc (R&D Systems) and cultured for 48 h. MTT (5 mg/ml in PBS) was added to each well, and the wells were incubated for 4 h. The purple-blue MTT formazan precipitate was dissolved in isopropanol containing 0.04N HCl. The optical density was measured at 595 nm using a microplate reader (Tecan).

2.5. Bromodeoxyuridine (BrdU) incorporation and TUNEL assay

U-251 and A172 cells cultured on coverslips were treated with BrdU (10 μM) for 30 min (U-251 cells) or 6 h (A172 cells) and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. To identify BrdU-incorporated cells, cells were pretreated with 4N HCl in PBS for 5 min at room temperature. Cells were then incubated with 0.2% Triton X-100 in PBS for 10 min and with 10% fetal bovine serum in PBS for 30 min to block nonspecific antibody binding. Cells were incubated with anti-BrdU antibody in PBS overnight at 4 °C, followed by incubation with Alexa Fluor 594-conjugated anti-mouse IgG antibody together with Alexa Fluor 488-conjugated anti-GFP antibody or Hochst 33258 (Thermo Fisher

Scientific) for 1 h at room temperature. After washing with PBS, cells were mounted in 90% glycerol containing 0.1% p-phenylenediamine dihydrochloride in PBS. Apoptotic cell death was detected by TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay using in situ cell death detection kit, TMR red (Roche) according to the manufacture's instruction. As a positive control, U-251 cells were treated with 1 mM H_2O_2 for 24 h [28]. Images were acquired using a Nikon Eclipse E800 microscope equipped with a 20x objective and a digital camera (Leica DC350F).

2.6. Immunoblotting

Cell lysates were separated by SDS-PAGE and electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore Corporation). The membrane was blocked with 3% low fat milk in Tris-buffered saline, and then incubated with primary antibodies. The primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL) detection kit (GE Healthcare).

2.7. Data analysis

Statistical significance was established using the two-sample Student's *t* test or the analysis of variance (ANOVA) and post hoc test (Bonferroni) (SPSS software version 16.0, IBM). $p < 0.05$ was considered significant.

3. Results

3.1. EphA2 is involved in EGF-induced cell proliferation in glioblastoma cells

To investigate whether EphA2 plays a role in EGF-triggered cell proliferation in glioblastoma, we used the glioblastoma cell line U-251. As reported previously in other cells [22, 29], expression of EphA2 in U-251 cells was down-regulated after long exposure (24 and 48 h) to ephrinA1-Fc (Fig. 1A). We found that long exposure to ephrinA1-Fc reduced the number of U-251 cells at 48 h after stimulation with EGF (Fig. 1B). We quantified cell proliferation by MTT assay, and found that treatment of U-251 cells with ephrinA1-Fc (48 h) significantly suppressed EGF-induced cell proliferation compared to control-Fc treatment (Fig. 1C). Previous studies have reported that ephrinA1-EphA2 interaction or EphA2 silencing can induce apoptosis [30, 31]. However, we could not detect apoptotic cells within 48 h after treatment of U-251 cells with ephrinA1-Fc (Fig. 1D, H_2O_2

was used as a positive control [28]). We further examined the effect of long exposure to ephrinA1 on EGF-stimulated cell proliferation by BrdU incorporation. After U-251 cells were stimulated with EGF in the presence of ephrinA1-Fc or control Fc for 20 h, they were labeled with BrdU for 30 min followed by staining with anti-BrdU antibody. Stimulation of U-251 cells with EGF increased the number of BrdU-positive cells. However, ephrinA1 treatment significantly suppressed EGF-induced BrdU incoporation (Fig. 2A and B). To confirm the involvement of EphA2 in EGF-stimulated U-251 cell proliferation, cells were transfected with shRNA against human EphA2 (shEphA2) or control shRNA (shControl) [18, 27] and then labeled with BrdU. Knockdown of EphA2 by shRNA significantly suppressed EGF-stimulated BrdU incorporation (Fig. 2C). We could not detect apoptosis in U-251 cells transfected with EphA2 shRNA (Fig. 2D). Taken together, these results suggest that EphA2 is required for the EGF-stimulated cell proliferation in U-251 cells.

To examine whether the involvement of EphA2 in EGF-induced promotion of cell proliferation is limited to U-251 cells, we used another glioblastoma cell line A172. A172 cells express similar endogenous level of EphA2 to U-251, and expression of EphA2 was down-regulated after long exposure to ephrinA1-Fc (Fig. 2E). Stimulation of A172 cells with EGF

increased the number of BrdU-positive cells, and ephrinA1 treatment significantly suppressed EGF-induced BrdU incorporation (Fig. 2F). Thus, the regulation of glioblastoma cell proliferation by EphA2 in response to EGF stimulation is not specific to U-251 cells.

3.2. EGF induces phosphorylation of EphA2 on S897 through the MEK/ERK/RSK pathway

Phosphorylation of EphA2 on S897 by Akt plays a key role in EphA2 ligand-independent glioblastoma cell migration and invasion [17, 19, 23]. Therefore, we next examined whether EphA2 is phosphorylated on S897 in response to EGF stimulation in U-251 cells using an antibody against S897-phosphorylated EphA2 (pS897-EphA2). We found that EGF stimulation resulted in marked phosphorylation of S897 EphA2. However, Akt inhibitor MK-2206 and its upstream PI3K inhibitor LY294002 had no effect on EGF-induced EphA2 phosphorylation on S897 (Fig. 3A). On the other hand, EGF-induced EphA2 S897 phosphorylation was completely suppressed by MEK inhibitors U0126 and PD980759 (Fig. 3B), suggesting that phosphorylation of EphA2 on S897 in U-251 cells occurs downstream of the MEK/ERK pathway. RSK is a downstream target of ERK [4, 5], and phosphorylation sites in many known substrates of Akt and RSK are similar

[32]. Therefore, we speculated that RSK is a candidate of kinases that induce EphA2 S897 phosphorylation. Our result showed that treatment of U-251 cells with RSK inhibitor BI-D1870 suppressed EGF-induced phosphorylation of EphA2 on S897 (Fig. 3C). To examine whether overexpression of RSK alone induces EphA2 S897 phosphorylation, we constructed HA-tagged wild-type RSK2, because U-251 cells express RSK2 among RSK members (data not shown). HEK293T cells were transfected with wild-type EphA2 (EphA2-WT) or S897A mutant (EphA2-SA) together with HA-RSK2, and the cell lysates were immunoblotted with anti-pS897-EphA2 antibody. Overexpression of EphA2-WT alone resulted in S897 phosphorylation, and it was enhanced by co-expression with RSK2. However, S897 phosphorylation was not detected in cells transfected with EphA2-SA and RSK2 (Fig. 3D). We also found that stimulation of A172 cells with EGF induced phosphorylation of S897 EphA2, and this phosphorylation was suppressed by BI-D1870 and U0126, but not by MK-2206 and LY294002 (Fig. 3E and F). These results suggest that EGF induces phosphorylation of EphA2 on S897 through the MEK/ERK/RSK pathway in glioblastoma cells.

3.4. EphA2 S897 Phosphorylation is required for the EGF-induced

promotion of cell proliferation

To examine whether EphA2 S897 phosphorylation by RSK is required for EGF-induced cell proliferation, we first examined the effect of RSK inhibitor BI-D1870 on EGF-stimulated BrdU incorporation in U-251 cells. We found that treatment of the cells with BI-D1870 significantly suppressed EGF-induced BrdU incorporation (Fig. 4A). Although RSK inhibition has been reported to induce apoptosis in other cancer cell types [33, 34], we could not detect apoptosis in U-251 cells under these conditions (Fig. 4B). To confirm the involvement of RSK in EGF-induced cell proliferation, we constructed an shRNA vector against human RSK2 (shRSK2), which effectively reduced the amount of exogenously expressed HA-RSK2 in HEK293T cells (Fig. 4C). U-251 cells were transfected with shRSK2 or shControl and labeled with BrdU. Knockdown of RSK2 by shRNA significantly suppressed EGF-stimulated BrdU incorporation (Fig. 4D). These results suggest that RSK2 is involved in EGF-induced cell proliferation. The RSK inhibitor also significantly suppressed EGF-induced promotion of BrdU incorporation in A172 cells (Fig. 4E).

We next transfected U-251 cells with EphA2-WT or EphA2-SA together with GFP for BrdU incorporation, and measured the number of BrdU-labeled GFP-positive cells in the total number of GFP-positive cells.

Overexpression of GFP alone had little effect on the EGF-induced promotion of BrdU incorporation. However, overexpression of EphA2-WT induced a significant increase in the number of BrdU-positive cells without EGF stimulation (Fig. 5A). In contrast, expression of EphA2-SA suppressed the EGF-induced promotion of BrdU incorporation. The immunofluorescence staining with anti-EphA2 antibody confirmed the expression of EphA2-WT or EphA2-SA in GFP-expressing cells (Fig. 5B). We also found that treatment of the cells with the RSK inhibitor BI-D1870 significantly suppressed EphA2-WT-induced BrdU incorporation (Fig. 5C). On the other hand, overexpression of RSK2 induced promotion of BrdU incorporation without EGF stimulation, and this was suppressed by co-expression with EphA2-SA (Fig. 5D). These results suggest that phosphorylation of EphA2 on S897 by RSK promotes cell proliferation.

4. Discussion

EphA2 is frequently overexpressed in glioblastoma and its expression level correlates with poor prognosis of patients. On the other hand, abnormal EGF receptor signaling is one of major factors for oncogenic progression of glioblastoma. However, the relationship between EGF receptor and EphA2 remains unknown. In this study, we show that

EphA2 plays a key role in EGF-stimulated glioblastoma cell proliferation through a ligand ephrin-independent manner. Previous studies reported that ligand-independent EphA2 S897 phosphorylation by Akt promotes glioblastoma cell migration and invasion. However, we found that stimulation of U-251 and A172 glioblastoma cells with EGF triggers phosphorylation of EphA2 on S897 through the MEK/ERK/RSK pathway and promotes cell proliferation. Thus, our results uncover a novel mechanism that regulates glioblastoma cell proliferation, and RSK is a key mediator of EGF receptor signaling to EphA2 in oncogenic responses.

Previous studies reported that Akt phosphorylates S897 of EphA2 in response to several growth factors and promotes cell migration and invasion [17, 19-21]. Phosphorylation of EphA2 by Akt also regulates stem cell like properties in glioma [23]. However, our data show that EphA2 phosphorylation on S897 in response to EGF stimulation is suppressed by MEK and RSK inhibitors, but not by PI3K and Akt inhibitors in glioblastoma cells. In addition, U-251 and A172 are PTEN-deficient cell lines, and Akt activity (T308 phosphorylation) is observed without growth factor stimulation in those cells. However, the basal level of EphA2 S897 phosphorylation is undetectable, and it is up-regulated by EGF stimulation. This result also supports our conclusion that Akt is not responsible for

phosphorylating S897 EphA2 in U-251 and A172 cells. Akt and RSK phosphorylate many known substrates at a similar motif [32]. In addition, Zhou et al. recently demonstrated that MEK and RSK inhibitors can block EphA2 S897 phosphorylation in many cancer cell types, and that RSK can directly phosphorylates S897 of EphA2 [25]. On the other hand, we recently reported that stimulation of MDCK cells with hepatocyte growth factor in three-dimensional culture phosphorylates S897 of EphA2 through the PI3K/Akt pathway and regulates epithelial morphogenesis [24]. Therefore, EphA2 phosphorylation on S897 by RSK or Akt may depend on the cellular context and/or functions.

Although genes of RSK family are not frequently mutated or amplified in malignant tumors, RSK is implicated in the regulation of cancer cell invasion and metastasis [7]. Among RSK family members, RSK1 and RSK2 have been reported to show promotion of cancer cell motility in various cancer cell types, including head and neck squamous cell carcinoma, colon adenocarcinoma, and prostate cancer cell lines, through cancer-specific mechanisms [35-37]. On the other hand, RSK1 and RSK2 promote proliferation of prostate and breast cancer cells [38, 39]. RSKs are known to phosphorylate several transcription factors and the cell cycle machinery, including c-Fos, $p27^{kip1}$, and Cdc25 [4-6]. In glioblastoma,

however, the roles of RSKs in cancer progression still remain unclear. In this study, we provide evidence that RSK is involved in cell proliferation in U-251 and A172 glioblastoma cells through phosphorylation of EphA2. Thus, our results suggest that EphA2 is one of key RSK substrates in the regulation of cancer cell proliferation. EphA2 phosphorylated on S897 also promotes cell migration and invasion in various types of cancer cells [17, 19-21, 23, 25]. Therefore, RSK may also regulate migration and invasion of glioblastoma cells by phosphorylating S897 of EphA2.

The mechanism by which ligand-independent EphA2 signaling promotes glioblastoma cell proliferation is unclear. In cholangiocarcinoma cells, overexpression of EphA2 has been reported to activate the mTORC1 pathway through a ligand-independent manner [40]. Therefore, we examined the involvement of EphA2 in the regulation of mTORC1 activity, which positively regulates cell proliferation in many cell types. However, both downregulation of EphA2 and treatment with the RSK inhibitor had little effect on the phosphorylation state of mTORC1 substrate ribosomal S6 kinase in response to EGF stimulation (data not shown). Further studies are required to elucidate how ligand-independent EphA2 signaling regulates glioblastoma cell proliferation.

5. Conclusion

In this study, we demonstrate a molecular link between EGF receptor and EphA2 in the regulation of glioblastoma cell proliferation. EGF stimulation results in phosphorylation of S897 EphA2 through the MEK/ERK/RSK pathway, leading to promotion of cell proliferation. On the basis of these findings, we propose that understanding the mechanism underlying the RSK-EphA2 axis may reveal new targets for EGF receptor-targeted brain cancer therapy.

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Abbreviations

EGF, epidermal growth factor; RSK, ribosomal S6 kinase; S897, serine 897; shRNA, short hairpin RNA; PI3K, phosphatidylinositol 3-kinase; MTT, 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide;

BrdU, bromodeoxyuridine.

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Figure legends

Fig. 1. EphA2 is involved in EGF-induced cell proliferation in U-251 cells. A. U-251 cells were treated with control-Fc or ephrinA1-Fc (1 μg/ml) for indicated times, and the cell lysates were analyzed by immunoblotting with antibodies against EphA2 and α-tubulin. B. U-251 cells were treated with control-Fc or ephrinA1-Fc (1 μg/ml) and stimulated with EGF (100 ng/ml) for 48 h. Scale bar, 100 μm. C. Cell proliferation was assessed by measuring MTT absorbance at 0 h and 48 h, and relative proliferation of cells were expressed as fold change in MTT absorbance relative to that at 0 h. Data are the means ± SEM of five independent experiments (**$p < 0.01$, one-way ANOVA, Bonferroni, EGF + control-Fc vs. EGF + ephrinA1-Fc). D. U-251 cells were treated with control-Fc or ephrinA1-Fc (1 μg/ml) and stimulated with EGF (100 ng/ml) for 48 h. As a positive control, U-251 cells were treated with 1 mM H_2O_2 for 24 h. Apoptotic cells were detected by TUNEL assay. Scale bar, 100 μm.

Fig. 2. EphA2 mediates EGF-stimulated BrdU incorporation. A. U-251 cells were treated with control-Fc or ephrinA1-Fc (1 μg/ml) together with EGF (100 ng/ml) for 20 h and labeled with BrdU (10 μM) for 30 min. Then cells were stained with anti-BrdU antibody and Hoechst 33258. Scale bar,

50 μm. B. The number of BrdU-positive cells was counted, and the percentage of BrdU-positive cells in the total cell number was shown. Data are the means ± SEM of four independent experiments (***$p < 0.001$, one-way ANOVA, Bonferroni). C. U-251 cells transfected with shControl or shEphA2 were treated with EGF (100 ng/ml) for 20 h and labeled with BrdU for 30 min. Then cells were stained with anti-BrdU and anti-GFP antibodies. The number of BrdU-positive and/or GFP-positive cells was counted, and the percentage of BrdU-labeled GFP-positive cells in the total number of GFP-positive cells (BrdU+GFP+/GFP+) was shown. Data are the means ± SEM of three independent experiments (*$p < 0.05$, one-way ANOVA, Bonferroni). D. U-251 cells were transfected with shControl or shEphA2, and apoptotic cells were detected by TUNEL assay. Scale bar, 50 μm. E. A172 cells were treated with control-Fc or ephrinA1-Fc (1 μg/ml) for 24 h, and the cell lysates were analyzed by immunoblotting with antibodies against EphA2 and α-tubulin. F. A172 cells were treated with control-Fc or ephrinA1-Fc (1 μg/ml) together with EGF (100 ng/ml) for 24 h and labeled with BrdU (10 μM) for 6 h. Then cells were stained with anti-BrdU antibody and Hoechst 33258. The number of BrdU-positive cells was counted, and the percentage of BrdU-positive cells in the total cell number was shown. Data are the means ± SEM of three independent

experiments (*p < 0.05, one-way ANOVA, Bonferroni).

Fig. 3. EGF induces EphA2 phosphorylation on S897 through the MEK/ERK/RSK pathway. A. U-251 cells were treated with MK2206 (1 μM) or LY294002 (20 μM) for 30 min and then stimulated with EGF (100 ng/ml) for 10 min. Cell lysates were analyzed by immunoblotting with the indicated antibodies. B. U-251 cells were treated with U0126 (20 μM) or PD98059 (20 μM) for 30 min and then stimulated with EGF (100 ng/ml) for 10 min. C. U-251 cells were treated with BI–D1870 (10 μM) or U0126 (20 μM) for 30 min and then stimulated with EGF (100 ng/ml) for 10 min. D. Cell lysates from HEK293T cells transfected with the indicated plasmids were immunoblotted with the indicated antibodies. E. A172 cells were treated with BI–D1870 (10 μM) or U0126 (20 μM) for 30 min and then stimulated with EGF (100 ng/ml) for 10 min. F. A172 cells were treated with MK2206 (1 μM) or LY294002 (20 μM) for 30 min and then stimulated with EGF (100 ng/ml) for 10 min. Cell lysates were analyzed by immunoblotting with the indicated antibodies.

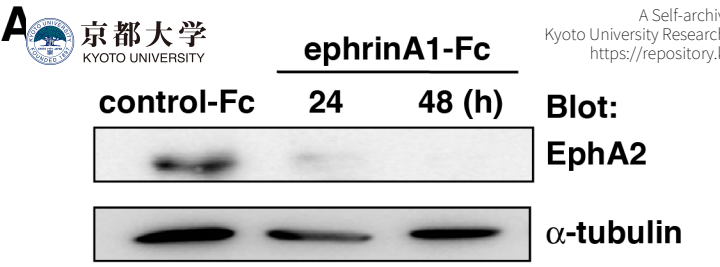
Fig. 4. RSK is involved in the EGF-induced promotion of cell proliferation. A. U-251 cells were treated with BI-D1870 (10 μM) for 30 min, and then

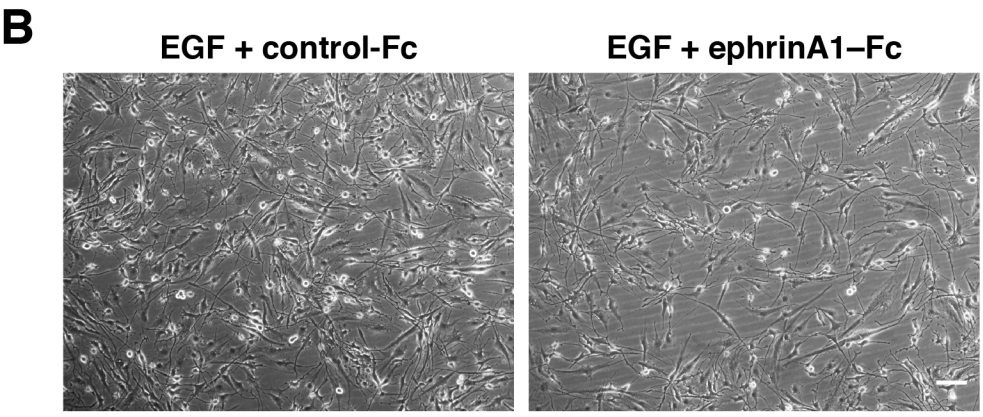
EGF (100 ng/ml) was added for 20 h. Cells were then labeled with BrdU for 30 min and stained with anti-BrdU antibody and Hoechst 33258. The number of BrdU-positive cells was counted, and the percentage of BrdU-positive cells in the total cell number was shown. Data are the means ± SEM of four independent experiments (*$p < 0.05$, student's t test). B. U-251 cells were treated with BI-D1870 for 30 min and stimulated with EGF (100 ng/ml) for 20 h. Apoptotic cells were detected by TUNEL assay. Scale bar, 100 μm. C. Cell lysates from HEK293T cells transiently transfected with shControl or shRSK2 were analyzed by immunoblotting with antibodies against HA and α-tubulin. D. U-251 cells transfected with shControl or shRSK2 were treated with EGF (100 ng/ml) for 24 h and labeled with BrdU for 30 min. Then cells were stained with anti-BrdU and anti-GFP antibodies. The number of BrdU-positive and/or GFP-positive cells was counted, and the percentage of BrdU-labeled GFP-positive cells in the total number of GFP-positive cells (BrdU+GFP+/GFP+) was shown. Data are the means ± SEM of three independent experiments (*$p < 0.05$, **$p < 0.01$, one-way ANOVA, Bonferroni). E. A172 cells were treated with BI-D1870 (10 μM) for 30 min, and then EGF (100 ng/ml) was added for 24 h. Cells were then labeled with BrdU for 6 h and stained with anti-BrdU antibody and Hoechst 33258. The number of BrdU-positive cells was

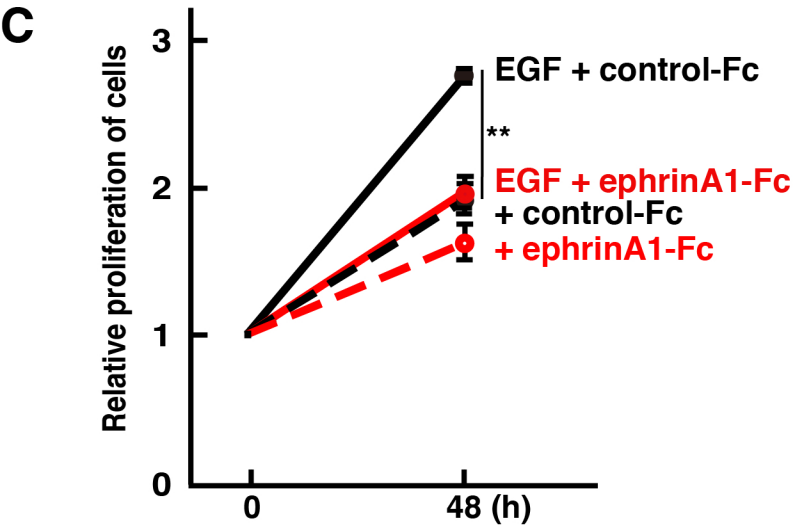
counted, and the percentage of BrdU-positive cells in the total cell number was shown. Data are the means ± SEM of six independent experiments (*p < 0.05, student's t test).

Fig. 5. EphA2 S897 phosphorylation is required for the EGF-induced cell proliferation. A. U-251 cells were transfected with GFP and the indicated plasmids and treated with EGF (100 ng/ml) for 20 h. Then cells were stained with anti-BrdU and anti-GFP antibodies. The number of BrdU-positive and/or GFP-positive cells was counted, and the percentage of BrdU-labeled GFP-positive cells in the total number of GFP-positive cells (BrdU+GFP+/GFP+) was shown. Data are the means ± SEM of four independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA, Bonferroni). B. U-251 cells transfected with GFP and the indicated plasmids were stained with anti-EphA2 antibody. Scale bar, 50 μm. C. U-251 cells transfected with GFP and EphA2-WT were treated with BI-D1870 for 20 h. Then cells were stained with anti-BrdU and anti-GFP antibodies. The number of BrdU-positive and/or GFP-positive cells was counted, and the percentage of BrdU-labeled GFP-positive cells in the total number of GFP-positive cells (BrdU+GFP+/GFP+) was shown. Data are the means ± SEM of five independent experiments (**p < 0.01, one-way

ANOVA, Bonferroni). D. U-251 cells were transfected with GFP and the indicated plasmids, and were stained with anti-BrdU and anti-GFP antibodies. The number of BrdU-positive and/or GFP-positive cells was counted, and the percentage of BrdU-labeled GFP-positive cells in the total number of GFP-positive cells (BrdU+GFP+/GFP+) was shown. Data are the means ± SEM of four independent experiments (**$p < 0.01$, one-way ANOVA, Bonferroni).







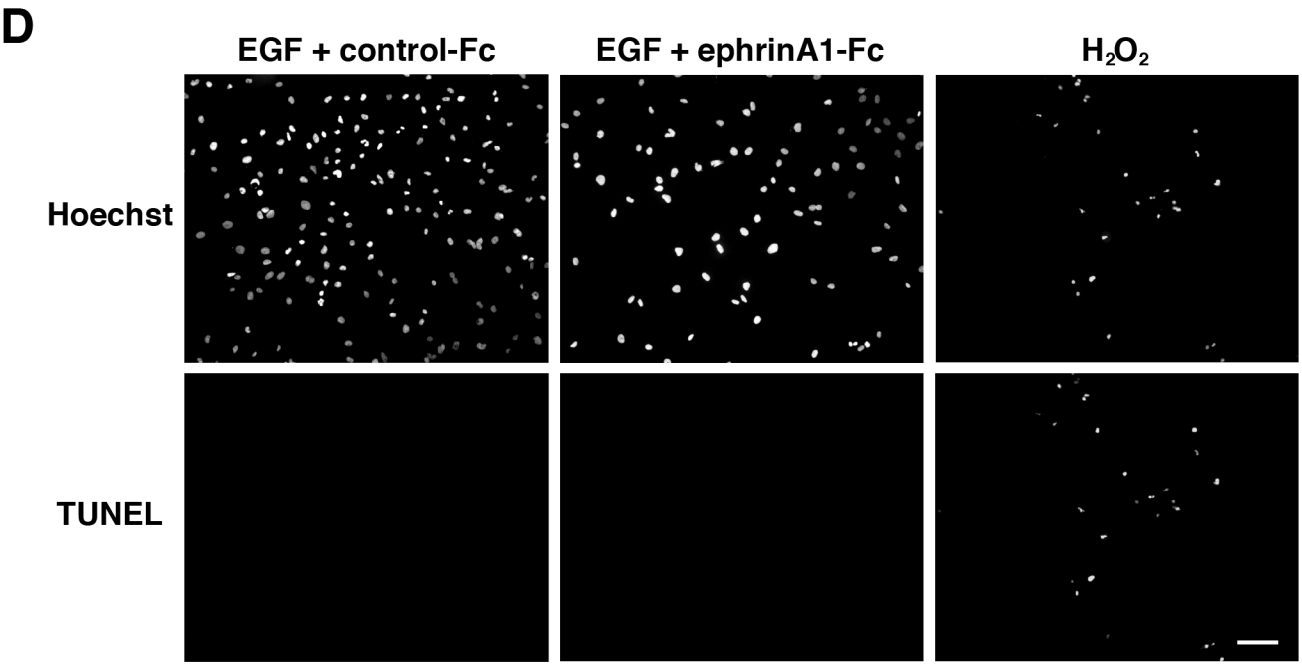
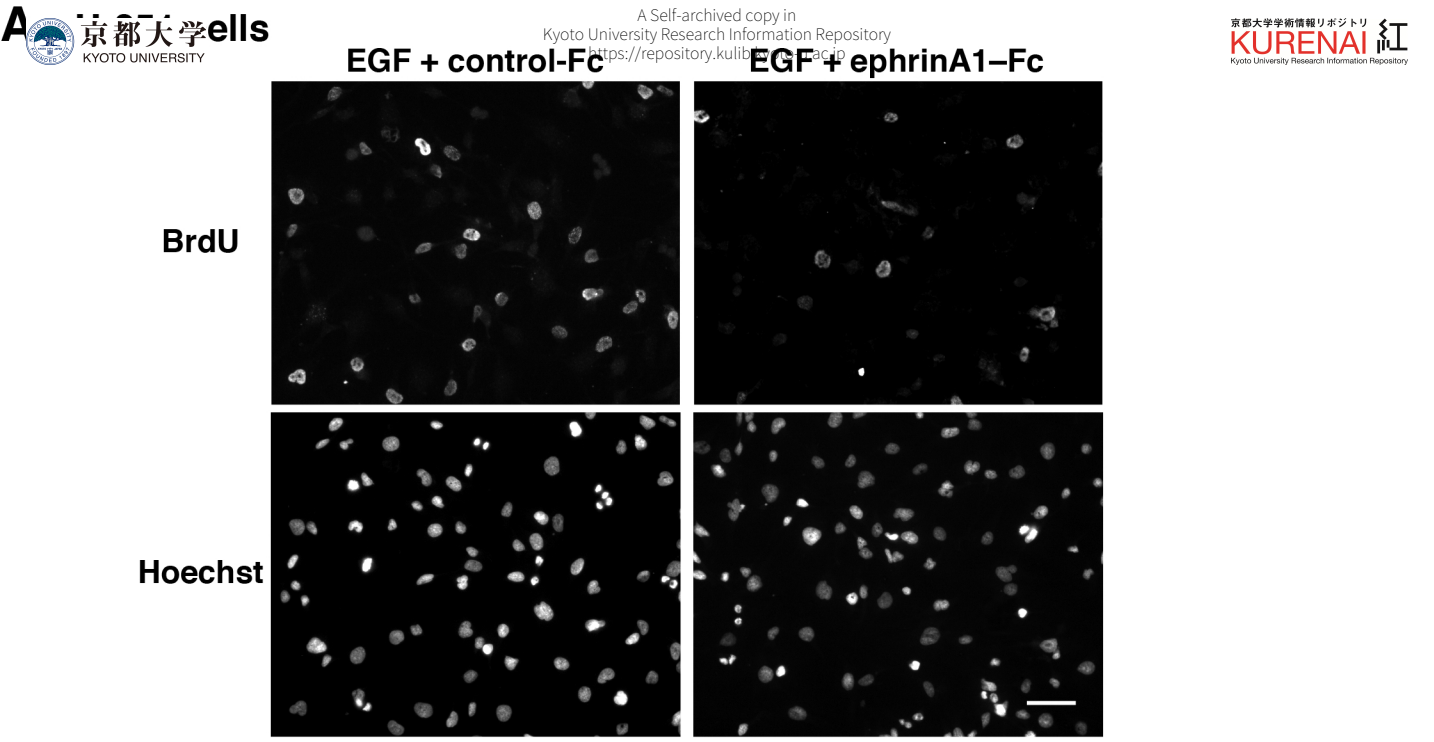
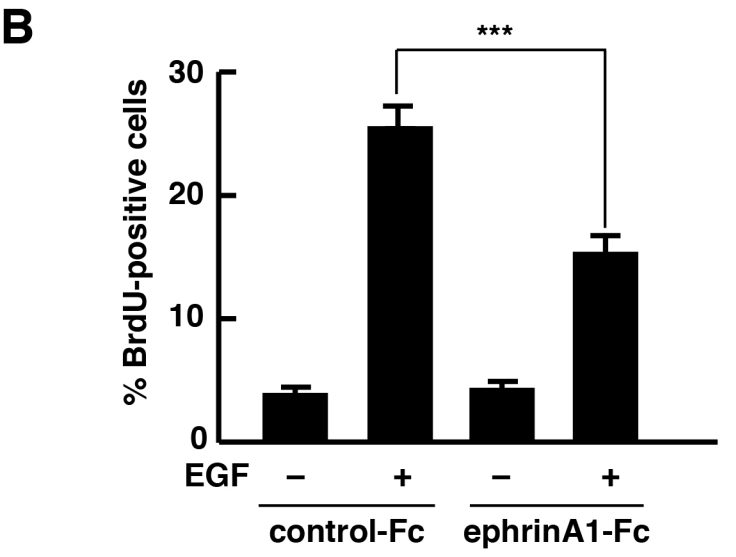
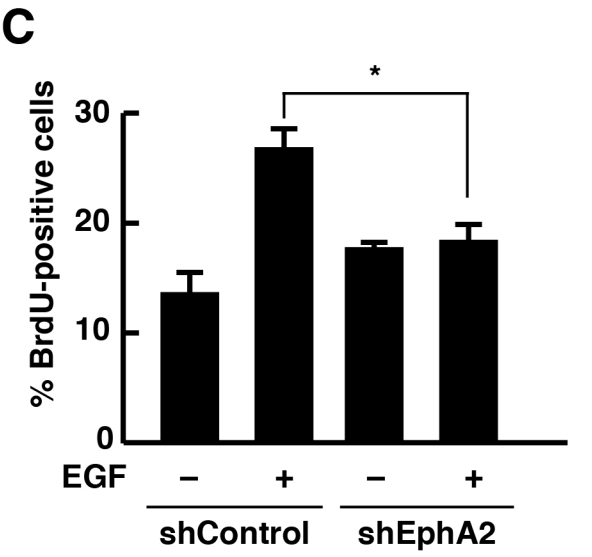
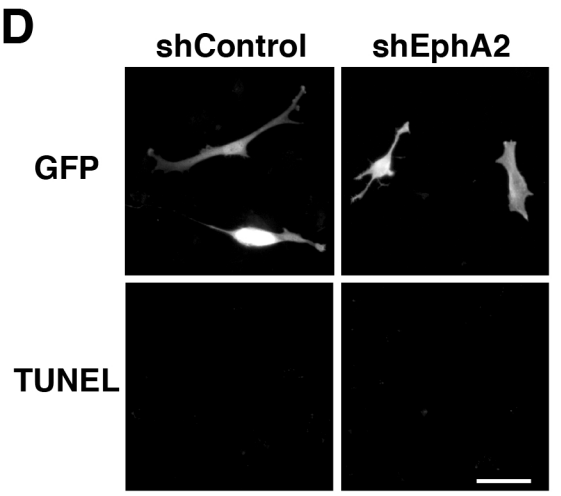


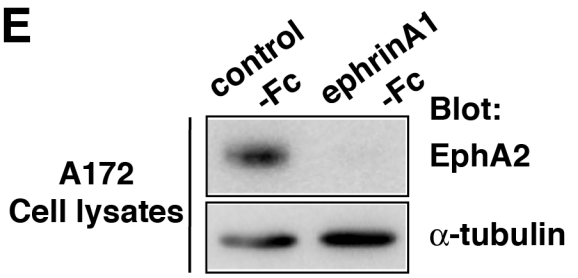
Figure 1











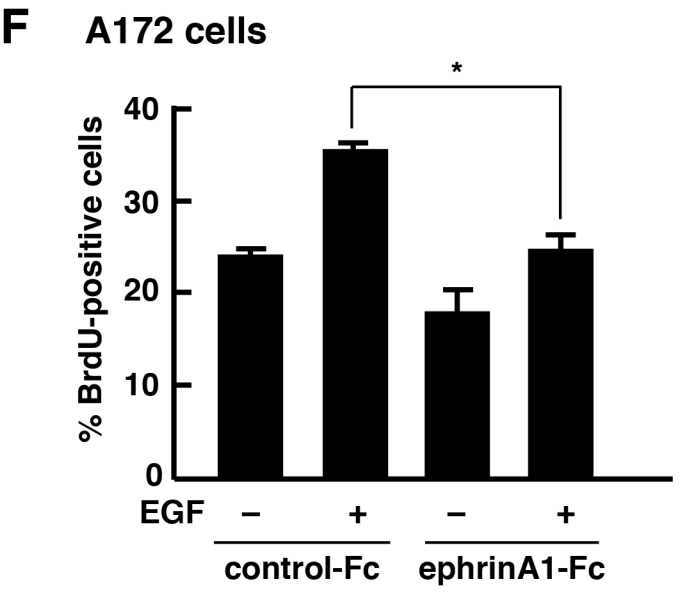


Figure 2

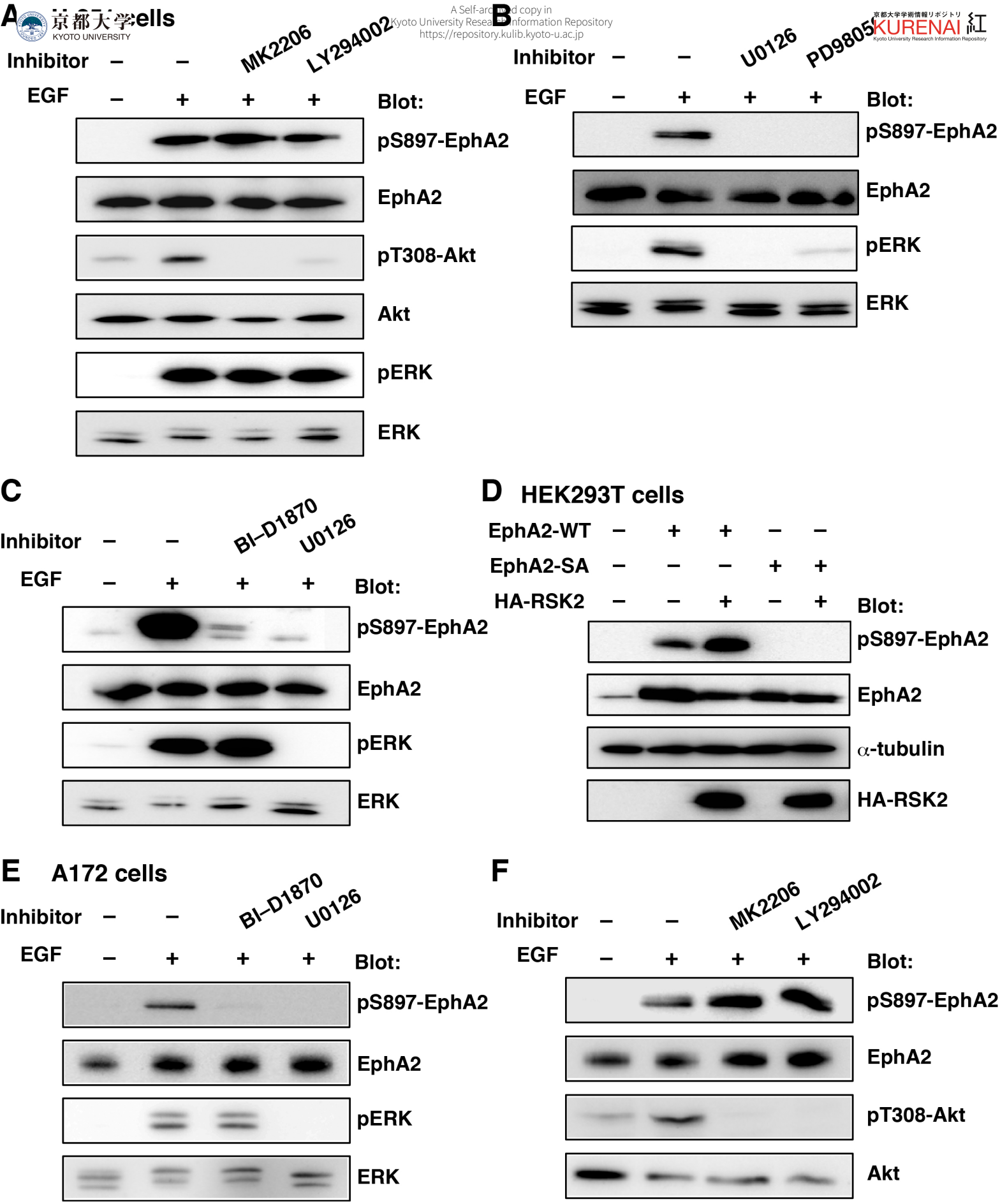
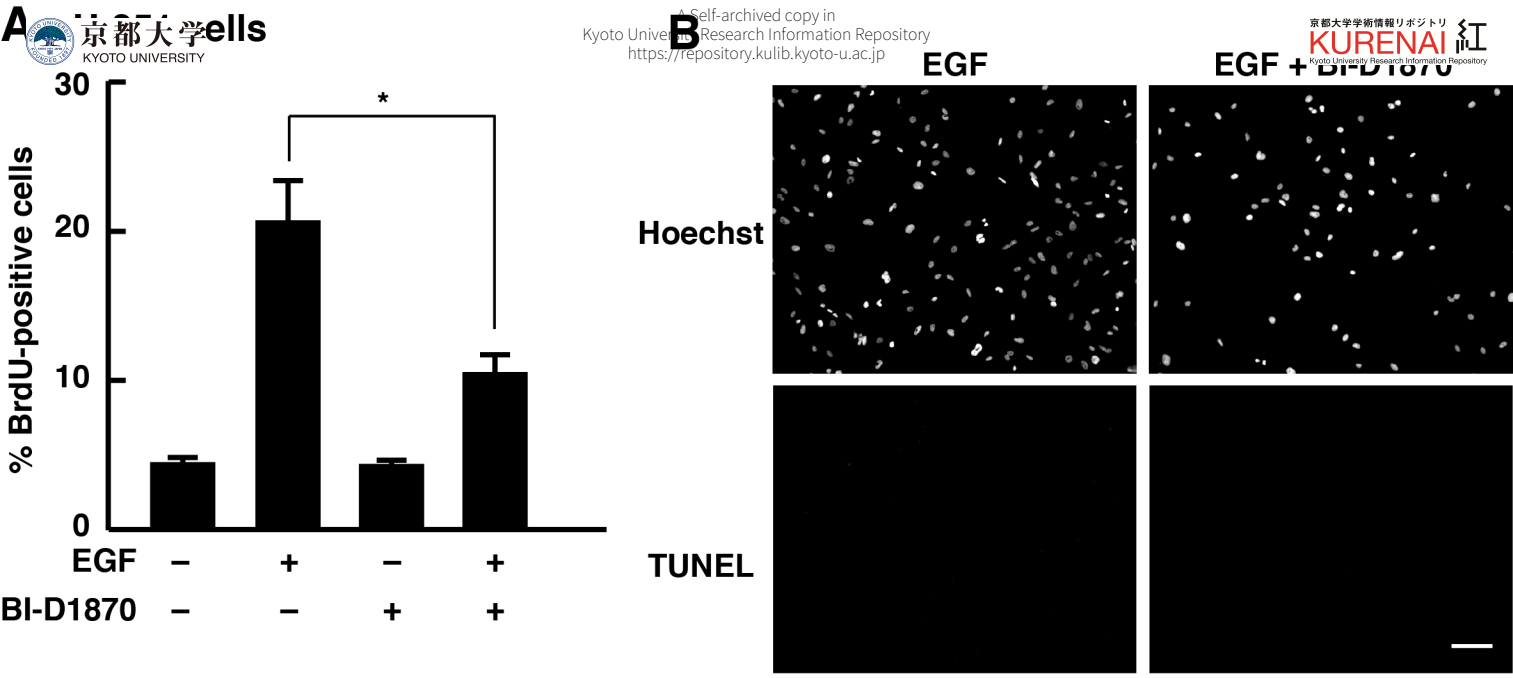
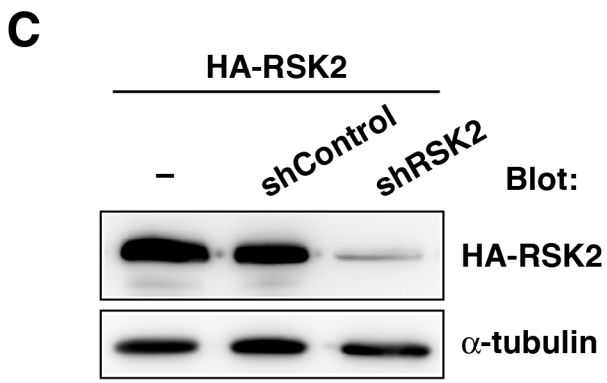
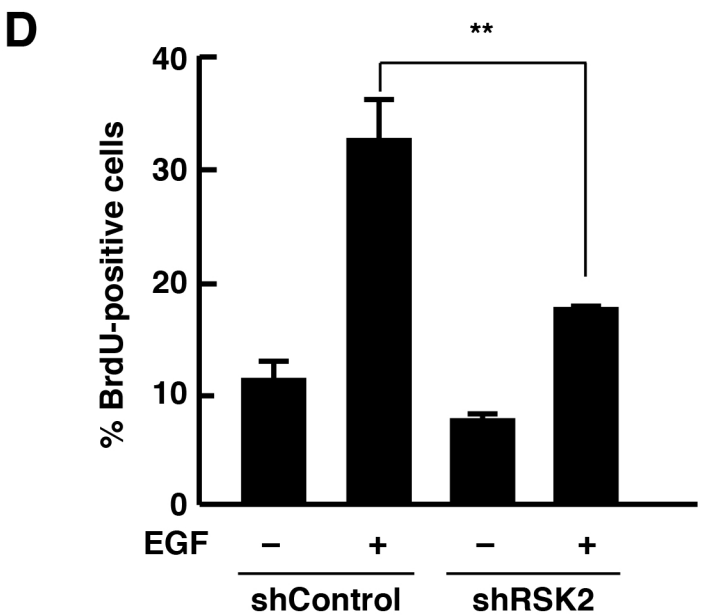


Figure 3







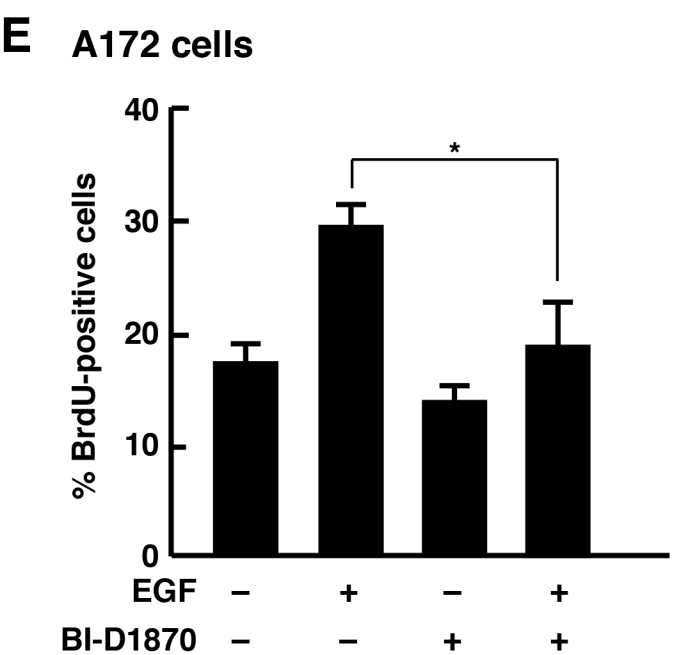
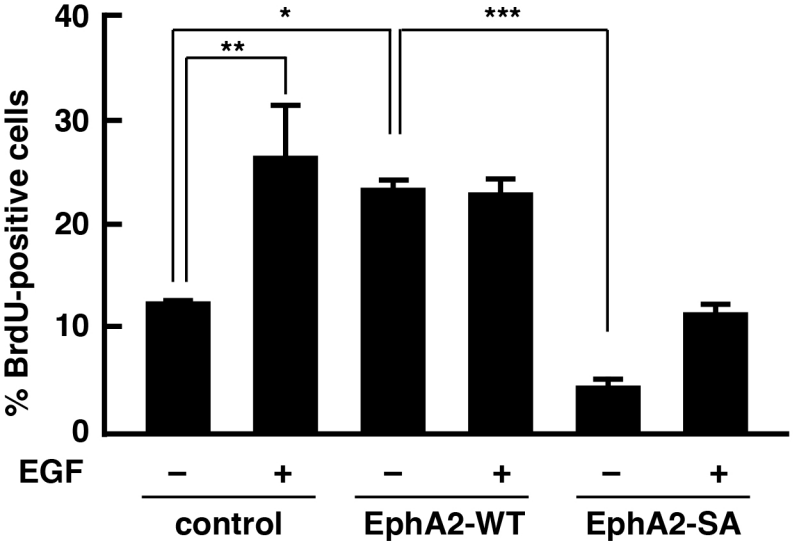
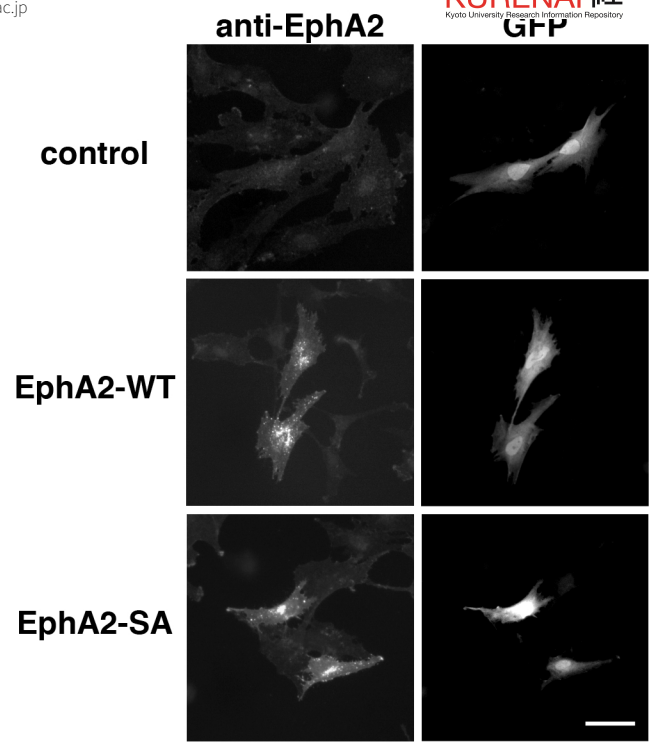
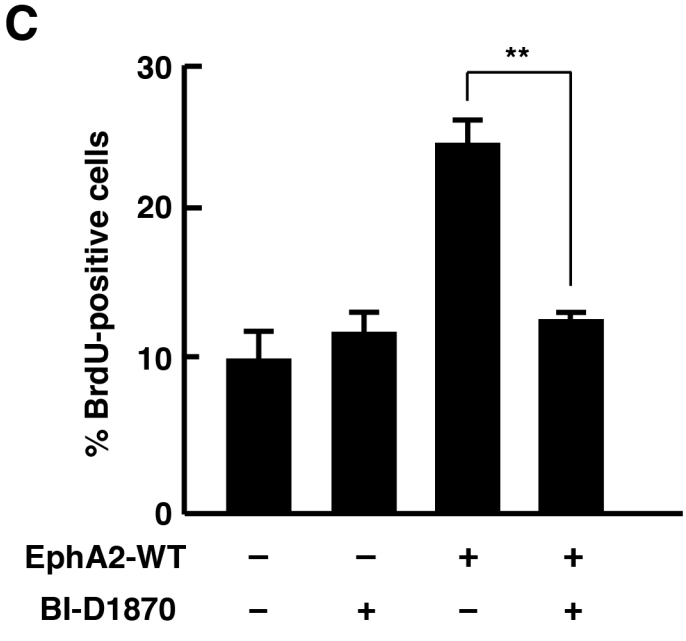


Figure 4







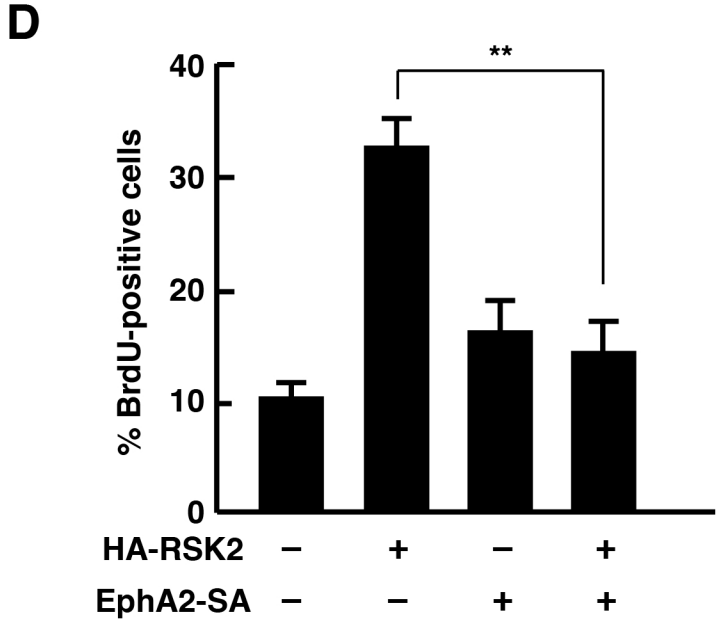


Figure 5